

## The T Cell-B Cell Interaction via OX40-OX40L Is Necessary for the T Cell-dependent Humoral Immune Response

By Eckhard Stüber and Warren Strober

*From the Mucosal Immunity Section, Laboratory of Clinical Investigations, National Institutes of Health, Bethesda, Maryland 20892-1890*

### Summary

Recent *in vitro* studies have established that activated B cells express OX40 ligand (L), a member of the tumor necrosis factor/nerve growth factor family of cytokines, and become stimulated to proliferate and secrete immunoglobulin (Ig) after cross-linking of OX40L by its counterreceptor OX40, which is expressed on activated T cells. In the present study we investigated the *in vivo* role of this receptor-ligand pair for the interaction of T and B cells in the course of the T-dependent B cell response against 2,4,6 trinitro-phenyl-keyhole limpet hemocyanin. First, we showed that OX40 is maximally expressed by T cells in the periarteriolar lymphoid sheath (PALS) 3 d after primary immunization. These OX40<sup>+</sup> cells are located in close proximity to antigen-specific, activated B cells. Second, we demonstrated that blocking of OX40-OX40L interaction with a polyclonal anti-OX40 antibody or with antibodies against certain peptide sequences within its extracellular domain resulted in a profound decrease of the anti-hapten IgG response, whereas the anti-hapten IgM response was grossly unchanged. Third, we showed that this antibody treatment leads to an inhibition of the development of PALS-associated B cell foci, whereas the formation of germinal centers remained intact. Finally, our data suggest that, whereas B cell memory development was not impaired by anti-OX40 administration, OX40-OX40L interaction seems to be crucial in the secondary immune response. We conclude from these data that the OX40-OX40L interaction *in vivo* is necessary for the differentiation of activated B cells into highly Ig-producing cells, but is not involved in other pathways of antigen-driven B cell differentiation such as memory cell development in the germinal centers.

The differentiation of mature B cells during the course of a T cell-dependent humoral immune response consists of Ig isotype switching and terminal differentiation into plasma cells or memory B cells. A number of studies have demonstrated that after initial stimulation through the antigen receptor and after contact help from activated T helper cells, B cells follow a branched differentiation pathway (1-3). In the spleen, the branching takes place in the periarteriolar lymphoid sheath (PALS)<sup>1</sup>. Some cells leave the PALS and enter the B cell follicle to form oligoclonal germinal centers in which they divide rapidly, undergo affinity maturation through somatic mutation, and eventually become memory B or plasma cells. In contrast, other cells

form PALS-associated B cell foci, which are largely responsible for the early primary humoral immune response (4).

A number of stimuli have been identified that are important for the initiation of the differentiation events occurring during a T-dependent, antigen-driven B cell differentiation (e.g., costimulation of T cells through CD28 [5-7]; CD40 ligand (L) L on activated T cells [8]), indicating their possible role in early B cell activation. However, with the exception of Ig isotype switching, which is made possible by stimulation through CD40 and guided by cytokines (9), the specific signals that drive a B cell towards terminal differentiation on the one hand or memory development on the other, have yet to be identified. In addition, it is unclear if the different B cell differentiation pathways mentioned above occur because of the presence of different precommitted mature B precursor cells (e.g., heat stable antigen (HSA)<sub>low</sub> versus HSA<sub>high</sub> cells; 10) or because a single population of B precursor cells receives different signals during their stay in the PALS.

<sup>1</sup>Abbreviations used in this paper: AFC, antibody-forming B cell; AP, alkaline phosphatase; CD40L, CD40 ligand; HSA, heat stable antigen; HRP, horseradish peroxidase; NGF, nerve growth factor; PALS, periarteriolar lymphoid sheath; TNP, 2,4,6 trinitro-phenyl.

To elucidate some of these questions we investigated the role of OX40-OX40L interaction—a relatively novel receptor-ligand pair—during the course of *in vivo* T-dependent B cell differentiation. OX40 is a member of the TNFR/nerve growth factor (NGF) R superfamily of receptors and is expressed on activated T cells (11, 12), whereas its ligand, OX40L, is found on activated B cells (12, 13). In previous studies we demonstrated that the cross-linking of OX40L on *in vitro* activated B cells resulted in an enhanced proliferation and a striking increase in Ig secretion (13). In addition, we showed that the latter effect was probably brought about by the effect of OX40L cross-linking on the state of activation of the Ig 3' enhancer (13).

In the present study we demonstrate that blocking of OX40-OX40L interaction *in vivo* by multiple injections of anti-OX40 antibody results in a profound decrease in the T cell-dependent antibody response, without the inhibition of germinal center formation or B cell memory development. These results thus suggest a critical role for OX40-OX40L interaction in driving activated B cells towards terminal differentiation into Ig-secreting plasma cells.

## Materials and Methods

**Cell Preparation, Culture Conditions and Flow Cytometric Analysis.** 6–9-wk-old female BALB/c mice (National Cancer Institute) were killed, their spleens removed and digested by collagenase (400 U/ml, Boehringer Mannheim, Mannheim, Germany) plus DNase (12.5 µg/ml; Boehringer Mannheim) at 37°C for 15 min. The resulting splenocyte suspension was depleted of RBC by hypotonic lysis with ACK lysing buffer (B&B Scott, West Warwick, RI). The cells collected after this treatment were applied to a T cell isolation column (Pierce Chemical Co., Rockford, IL) according to the manufacturer's protocol. The resulting cell population was 85–90% positive for CD3 as determined by flow cytometry using the FACScan® (Becton Dickinson & Co., San Jose, CA).

T cells were incubated on anti-CD3ε (145-2C11)-coated 12-well plates (Costar Corp., Cambridge, MA) at 37°C, and 6% CO<sub>2</sub> in RPMI 1640 was supplemented with 10% FCS, 5% NCTC 109 media (Whittaker M. A. Bioproducts, Walkersville, MD), 20 mM Hepes (NIH media unit), 2 mM L-glutamine (NIH media unit), 50 µM β-mercaptoethanol (Sigma Chemical Co., St. Louis, MO), and 10 U/ml penicillin/100 µg/ml streptomycin (GIBCO BRL, Gaithersburg, MD).

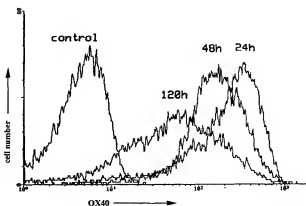
After the indicated periods of time, T cells were analyzed for OX40 expression by flow cytometry using the FACScan® and the biotinylated polyclonal anti-OX40 antibody (described below), as well as streptavidin-PE, anti-CD4-FITC and anti-CD8-FITC antibodies (PharMingen, San Diego, CA).

**Preparation of 2,4,6-Trinitro-Phenyl Protein Derivatives.** TNP-(2,4,6-trinitro-phenyl) KLH or TNP-BSA were prepared as follows. 20 mg lyophilized KLH (Pierce Chemical Co.) or 4.8 mg BSA (Sigma Chemical Co.) was dissolved in 4 ml potassium borate buffer (0.25 M, pH, 9.2), and 3 or 6 mg TNBS (2,4,6-trinitrobenzyl sulfonic acid; Sigma Chemical Co.) and 16 µl sodium carbonate (1 M) were added. The reaction was allowed to take place overnight, after which the protein derivatives were dialyzed against PBS, pH: 7.4. TNP protein derivatives were frozen at –20°C until use. TNP-LPS was purchased from Sigma Chemical

Co. and TNP-Ficoll was kindly provided by Dr. John Inman (Laboratory of Immunology, National Institutes of Health [NIH]).

**Preparation of OX40 Peptides.** The following two peptide sequences of the extracellular domain of the murine OX40 protein (12) were selected: P-OX-1<sub>106-123</sub> and P-OX-2<sub>127-164</sub>. These peptides were synthesized by the Fmoc method (Dr. John Coligan, Laboratory of Molecular Structure, NIH) and conjugated to maleimide-activated KLH according to the manufacturer's protocol. The conjugates were dialyzed against PBS and used for the production of antipeptide antibodies.

**Preparation of Anti-OX40 Antibodies.** 100 µg OX40-Ig fusion protein (12) or 2.5 mg P-OX-1- or P-OX-2-KLH in CFA was subcutaneously injected into 8–10-wk-old New Zealand White (NZW) rabbits. Serum was collected after 4 wk and analyzed for antibody titers by ELISA. In the case of the P-OX peptides, animals were boosted with 1 mg P-OX-KLH twice before antisera were drawn after 7 wk. Antisera were subsequently treated with 45% saturated ammonium sulfate (Sigma Chemical Co.); the precipitate was dissolved in PBS and dialyzed against PBS overnight at 4°C. The resulting Ig-enriched antisera were subsequently affinity purified as follows. First, the antisera were depleted of anti-human Fcγ1 antibodies (only in the case of antisera against OX40-Ig) by applying them to a column (model C10/10; Pharmacia Biotech, Piscataway, NJ) containing human IgG1 (Calbiochem-Novabiochem Corp., San Diego, CA)-coated, CNBr-activated Sepharose beads (Pharmacia Biotech). Second, the resulting antisera were run over another C10/10 column, that contained OX40-Ig-coated Sepharose beads; bound antibodies were eluted from the column with a 100-mM glycine, pH 2.5, wash, which was collected in 1 M Tris-HCl, pH 7.4. Third, after another precipitation step with 45% saturated ammonium sulfate, the precipitate was dissolved and dialyzed as mentioned above. The resulting polyclonal rabbit anti-murine OX40 or P-OX antibody-containing solutions were checked for their ability to bind to OX40 by ELISA and Western blot and were found to contain minimal (<5%) anti-human IgG1 activity (in the case of anti-OX40-Ig antibodies). The purity was assessed by SDS-PAGE analysis and the protein concentration was determined using the Bradford assay (14). The purified antibodies were frozen at –20°C until use. For immunohistochemistry and FACS® analysis, the anti-OX40



**Figure 1.** Expression of OX40 by *in vitro* activated T cells. Splenic T cells were incubated on anti-CD3-coated 12-well plates and examined for the expression of OX40 after the indicated incubation periods by flow cytometry using the biotinylated rabbit anti-OX40 antibody (see Materials and Methods) or rabbit IgG as control. 2.4G2 was used to eliminate binding to Fc-receptors. Results represent two independent experiments.

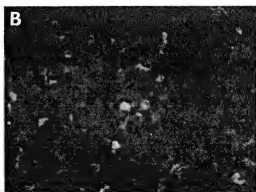
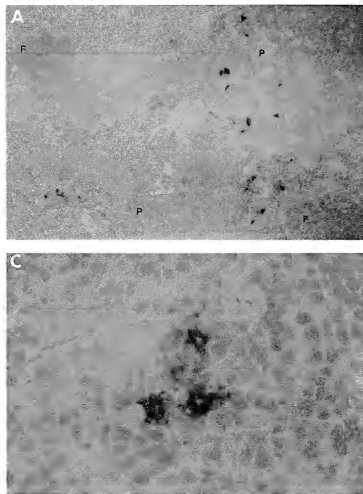
antibody and the rabbit control IgG (Calbiochem-Novabiochem Corp.) were biotinylated using Biotin-X-NHS (Boehringer Mannheim) according to the manufacturer's protocol.

**In Vivo Protocol for Hapten-specific Antibody Production.** TNP-KLH, TNP-Ficoll, or TNP-LPS (100 µg each in CFA) was injected intraperitoneally into 6–8-wk-old female BALB/c mice (NCI, NIH). The animals were subsequently administered 200 µg rabbit anti-OX40 or rabbit IgG as a control (Calbiochem-Novabiochem Corp.) daily on days 1–6 after immunization. These antibody concentrations were found to be effective as previously described (13). Serum was collected on days 7, 10, 17, and 24. Serum concentrations of anti-TNP-specific antibodies were determined as described below.

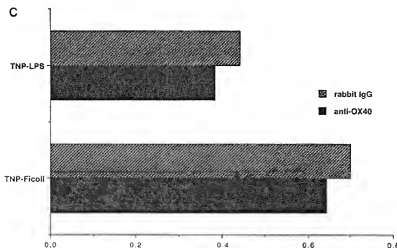
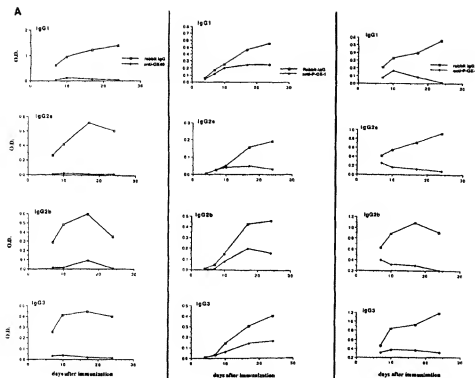
**Assay for Anti-TNP-Specific Ig.** 0.3 mg/ml TNP-BSA in carbonate buffer, pH 9.0, was bound to 96-well ELISA plates, that were subsequently blocked with 1% BSA in PBS, after which murine sera from the anti-OX40-treated or from the control mice, diluted in PBS containing 1% BSA (from 1:100 to >1:100,000), were incubated for 2 h at 37°C. The plates were then washed and goat anti-murine IgM, IgG1, IgG2a, IgG2b, IgG3, IgA, or IgE conjugated to alkaline phosphatase (AP) (1:500; Southern Biotechnology Associates, Birmingham, AL; IgE-AP, PharMingen) was added. Color reactions were performed using AP substrate (Sigma Chemical Co.) in diethanolamine buffer, pH: 8.5. To

compare different specimens, the OD reading at 490 nm from the 1:400 (or 1:1000 in the case of IgG1) dilution was evaluated. This was demonstrated to be well within the linear part of the titration curve.

**Immunohistochemistry.** Mice treated intraperitoneally with TNP-KLH (100 µg in CFA) and anti-OX40 antibodies (200 µg for 6 d), rabbit IgG or PBS as controls were killed on day 7 or 11, respectively. The spleens were removed, frozen in OCT-Freezing Media (Miles, Elkhart, IN) on dry ice and kept at -80°C. For the staining with anti-OX40 antibodies, mice were inoculated with 100 µg i.v. TNP-KLH and the spleens were removed on days 2, 3, 4, 5, 7, and 10. Frozen tissues were subsequently cut into 6–9-µm sections, transferred to glass slides, dried overnight, and kept at -80°C until use. Subsequently, the frozen sections were fixed in cold acetone for 5 min, rehydrated in PBS for 10 min, and blocked with 1% BSA and 3% goat serum for 60 min. This step was followed by a 60-min incubation period with biotinylated anti-OX40, anti-CD4-FITC, or biotinylated anti-IgD (PharMingen). PNA-biotin (Pierce Chemical Co.) was used to detect germinal centers. As secondary substances, streptavidin-AP (Boehringer Mannheim), streptavidin-PE, and streptavidin-horseradish peroxidase (HRP) (PharMingen) or streptavidin-Texas red (Amersham, Arlington Heights, IL) were used. Anti-TNP-specific B cells were visualized by a TNP-AP conjugate, that was prepared



**Figure 2.** After systemic immunization, OX40 is expressed by CD4<sup>+</sup> T cells, which are in close proximity to specific APCs. BALB/c mice were injected with 100 µg TNP-KLH and their spleens removed and frozen on dry ice 3 d after immunization. Immunohistochemistry was performed on frozen sections as described in Materials and Methods. (A) Cryosections were stained with biotinylated anti-OX40, washed, and subsequently incubated with streptavidin-AP. The Vector red substrate kit for AP was used for color reaction and the tissue was counterstained with 5% methyl-green. OX40<sup>+</sup> cells are located in the inner and outer PALS (P). No OX40<sup>+</sup> cells were detected in B cell follicles (F). (B) Splenic frozen sections were incubated with rat anti-mouse CD4-FITC and the biotinylated anti-OX40 antibody. After that, a second incubation with streptavidin-Texas red followed. Fluorescence microscopy was performed on the Zeiss Axiophot microscope. CD4<sup>+</sup> cells appear green, whereas CD4/OX40 double-positive cells (green and red) appear yellow. (C) Sections were incubated with biotinylated anti-OX40 and TNP-AP conjugate, washed and incubated with streptavidin-HRP. The Vector red substrate kit for AP (red) and the DAB kit (brown/black) was used for color reactions. Photomicrograph depicts OX40<sup>+</sup> cells (brown/black) in close proximity to antibody-forming B cells (red). (A) ×200, (B) ×400, and (C) ×630.



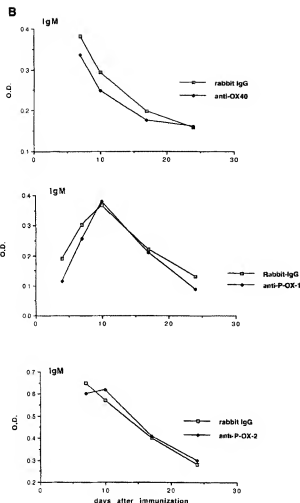
done separately. (B) Anti-TNP-specific IgM in response to TNP-KLH was determined in the same mice as in A. The results represent the OD<sub>490</sub> readings of two independent experiments for each of the three antibodies. (C) Anti-TNP-specific IgG 3 wk after immunization with TNP-Ficoll or TNP-LPS was determined. No significant difference in all IgG subclasses between the anti-OX40 and the rabbit IgG control group was detected. Results depict the OD<sub>490</sub> readings for total anti-TNP-IgG for two independent experiments for each antigen.

as described elsewhere (15). The color reaction was induced using either Vector red substrate for AP or the diaminobenzoic-acid substrate for HRP (Vector Laboratories, Inc., Burlingame, CA). Slides were counterstained with 5% methyl-green for 3 min, air-dried, and mounted in Permount (Fisher Scientific, Fair Lawn, NJ). Fluorescence microscopy was performed on the Axiophot microscope (Zeiss, Oberkochen Germany). The resulting image was printed on a color video printer (model UP-500; Sony, Tokyo, Japan).

## Results

**Expression of OX40 In Vitro and In Vivo.** In initial studies, we determined the time course of expression of OX40 on activated T cells in vitro. Splenic T cells were stimulated with plate-bound anti-CD3, and flow cytometry on the cells was performed using the biotinylated rabbit anti-murine OX40 antibody (see Materials and Methods) at 12, 24, 48,

**Figure 3.** Administration of three different anti-OX40 antibodies inhibit the T cell-dependent IgG but not IgM production and do not alter T-independent responses. BALB/c mice were injected with 100  $\mu$ g i.p. TNP-KLH (T cell-dependent), TNP-Ficoll (TI-2), and TNP-LPS (TI-1) in CFA and either treated with anti-OX40 antibodies, PBS, or control rabbit IgG (200  $\mu$ g on days 1–6). Serum was collected at the indicated time points and anti-TNP-specific Ig was measured as described in Materials and Methods. No difference was observed between the rabbit IgG and the PBS treated group (data not shown). (A) Anti-TNP-specific IgG of all subclasses in response to TNP-KLH after the administration of anti-OX40 (left), anti-P-OX-1 (middle), anti-P-OX-2 (right), or rabbit IgG was determined as described in Materials and Methods. TNP-specific IgA and IgE responses were below the detection limit. The results represent the OD<sub>490</sub> readings of two independent experiments for each of the three antibodies. ODs can only be compared within each column because experiments were



72, 96, and 120 h. OX40 was absent in resting cells, but was expressed on activated T cells as early as 12 h after stimulation, and as depicted in Fig. 1, the peak expression was observed after 24 h. In addition, the expression of OX40 remained upregulated throughout the investigated 5-d incubation period. Finally, both CD4<sup>+</sup> and CD8<sup>+</sup> T cells expressed OX40 after stimulation with anti-CD3 *in vitro* (data not shown); this finding is in agreement with those of Baum et al. (16), but is in contrast to the initial description of OX40 expression in the rat, in which such expression is restricted to CD4<sup>+</sup> T cells (11).

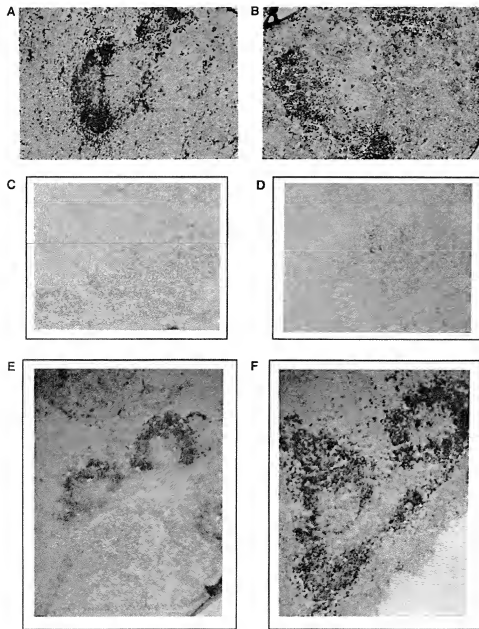
To determine the *in vivo* kinetics and expression pattern of OX40 on activated T cells, we immunized female BALB/c mice with TNP-KLH and performed immunohistochemistry on frozen spleen sections 2, 3, 4, 5, 7, and 10 d after immunization. Whereas no cells in tissues of unimmunized mice and only a few cells stained positive for OX40 2 d after immunization (data not shown), in tissues obtained from mice 3 d after immunization, OX40 was expressed by many cells within the inner and outer parts of the splenic PALS, but not within B cell follicles, as shown in Fig. 2 A. No significant OX40 expression was observed after day 4. A similar

expression pattern with even higher numbers of OX40<sup>+</sup> cells was observed after a secondary immunization (data not shown). Furthermore, as illustrated in Fig. 2 B, OX40 was predominantly expressed on CD4<sup>+</sup> T cells, with few CD8<sup>+</sup> T cells expressing OX40 (data not shown). We also investigated the location of OX40<sup>+</sup> T cells in relation to TNP-specific antibody-forming B cells (AFCs) after primary immunization with TNP-KLH. Previous studies *in vivo* have shown that TNP-specific AFCs in the PALS express OX40L (12). As illustrated in Fig. 2 C, some OX40<sup>+</sup> T cells and antigen-specific B cells lie in close proximity to each other, suggesting a possible interaction through OX40 and its ligand on the B cell.

**Blocking the OX40–OX40L Interaction *In Vivo* Results in a Profound Inhibition of T Cell-dependent Antibody Production.** We next investigated the role of the OX40–OX40L interaction during the course of a primary humoral immune response against the T cell-dependent antigen, TNP-haptenated KLH. In these studies, we first generated specific polyclonal rabbit anti-OX40 antibodies using either the OX40-Ig fusion protein or two 18-mer peptides (P-OX-1<sub>106–123</sub> and P-OX-2<sub>147–164</sub>) derived from the predicted amino acid sequence of the extracellular domain of murine OX40 (12) to immunize NZW rabbits. The resulting rabbit antisera were purified using affinity chromatography as described in Materials and Methods. The specificity of these antibodies was confirmed by the inhibition of the stimulatory effects of OX40-Ig (described in 13) on B cell functions *in vitro* (data not shown). As depicted in Fig. 3 A, the injection of rabbit IgG together with TNP-KLH resulted in a strong anti-TNP-IgG response that did not differ from the response to this antigen in PBS-treated mice (data not shown). However, sera from mice that were treated with the anti-OX40 antibody exhibited virtually no antihapten-specific IgG antibody response of any of the mice IgG subclasses. The anti-P-OX-2 antibody also resulted in profound inhibition, whereas anti-P-OX-1 only inhibited this response by 50–75%, depending on the subclass. It is interesting to note that, as shown in Fig. 3 B, the anti-TNP IgM response was not altered after treatment with anti-OX40, anti-P-OX-1, or anti-P-OX-2.

In complementary studies, we investigated whether anti-OX40 treatment affected T cell-independent B cell responses. Thus, we challenged mice with TNP-Ficoll or TNP-LPS, which are known T cell-independent (TI-2 and TI-1, respectively) B cell stimuli, and also injected these mice with anti-OX40 antibody or control rabbit IgG. As shown in Fig. 3 C, the TNP-specific IgG response was not significantly changed by the anti-OX40 treatment. Finally, we performed *in situ* staining for cytokines on frozen spleen sections (IL-2, IL-4, IL-5, and IFN- $\gamma$ ) from control and anti-OX40-treated mice and found no decrease in cytokine-secreting T cells in the anti-OX40-treated mice compared to control mice (data not shown).

**Immunohistochemical Studies after Anti-OX40 Treatment.** In the spleen, antigen-driven B cell differentiation takes place at two distinct sites: in the PALS-associated B cell foci, in which isotype switching and the development of B



**Figure 4.** The formation of PALS-associated B cell foci but not of germinal centers after systemic immunization with TNP-KLH is inhibited by the administration of anti-OX40. BALB/c mice were injected with 100  $\mu$ g i.p. TNP-KLH in CFA. Spleens were removed 7 or 11 d after immunization and immunohistochemistry was performed as described in Materials and Methods. (A and B) Cryosections from spleens 7 d after the immunization were incubated with TNP-AP and biotinylated anti-IgD. Streptavidin-HRP was employed as secondary reagent. Vector Red (red) and DAB (brown/black) was used for color reactions. Photomicrographs depict TNP-specific AFCs (red) located at the outer limit of the PALS (P) after treatment with control rabbit IgG (A), whereas the administration of anti-OX40 antibodies results in the appearance of far fewer and more scattered TNP-specific AFCs (B). Arrow indicates an early germinal center containing antigen-antibody complexes. Photomicrographs illustrate results of three independent experiments ( $\times 200$ ). (C and D) Cryosections from spleens 11 d after immunization were incubated with biotinylated PNA. Streptavidin-HRP was used as secondary reagent and DAB (brown/black) was employed for color reactions. Typical PNA<sup>+</sup> germinal centers appeared in control-treated mice (C) as well as in anti-OX40-treated mice (D). Photomicrographs illustrate results of three independent experiments. (E and F) Cryosections from spleens 11 d after immunization were incubated with biotinylated anti-IgD. Streptavidin-HRP was used as secondary reagent and DAB (brown/black) was employed for color reactions. Typical IgD<sup>+</sup> germinal centers appeared in control-treated mice (E) as well as in anti-OX40-treated mice (F). Photomicrographs illustrate results of three independent experiments. (A, B, E, and F)  $\times 200$ ; (C and D)  $\times 400$ .

tion. Typical IgD<sup>+</sup> germinal centers appeared in control-treated mice (E) as well as in anti-OX40-treated mice (F). Photomicrographs illustrate results of three independent experiments. (A, B, E, and F)  $\times 200$ ; (C and D)  $\times 400$ .

cells secreting high amounts of Ig occurs (days 3–10 after immunization), and in germinal centers, which are the site of isotype switching, as well as somatic mutation and B memory cell development (days 7–21; 4). Using TNP-haptenated AP, we followed the appearance of TNP-specific B cells in the PALS after *in vivo* challenge with TNP-KLH. As shown in Fig. 4 A, in control mice 7 d after the i.p. injection of TNP-KLH in CFA, the formation of clustered antigen-specific B cell foci in the outer PALS was observed. In contrast, as illustrated in Fig. 4 B, in mice treated with anti-OX40, whereas antigen-specific B cells also oc-

curred, they were much less frequent and they did not form the typical focal structure in the PALS. On the other hand, as shown in Fig. 4, C–F, no differences between the control and anti-OX40-treated mice could be detected in germinal center development evaluated at day 11 after TNP-KLH injection as assessed by anti-IgD and PNA staining.

**The Effect of Anti-OX40 Treatment during a Secondary Immune Response.** In a final set of experiments, we investigated the relevance of the OX40–OX40L interaction during a primary immune reaction for the importance of this

interaction during a secondary T cell-dependent humoral immune response. For this purpose we first immunized mice with TNP-KLH and treated them with either rabbit IgG or with anti-OX40 antibodies; the results of these experiments were shown above. After 4 mo the mice were reimmunized and either not treated with any antibody at all or treated as described for the primary immunization. As illustrated in Fig. 5 (*top*), the secondary immune response after treatment with anti-OX40 at the time of the primary antigenic challenge only was not altered in comparison to control-treated mice. However, as depicted in Fig. 5 as well (*bottom*), when these animals were also treated with anti-OX40 antibodies during the secondary immunization, a complete inhibition of the secondary IgG1 response was observed.

## Discussion

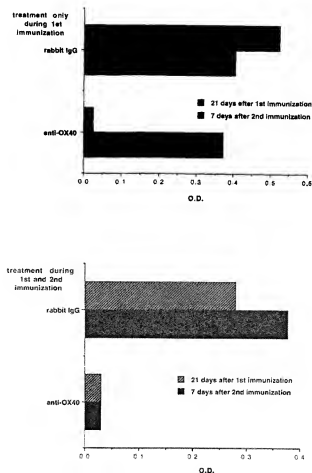
OX40 is a member of the NGFR/TNFR superfamily of receptors expressed on activated T cells which, upon cross-linking, results in enhanced T cell proliferation and cytokine secretion (11, 16, 17). Its ligand, OX40L, is found on activated B cells and, in a recent study we showed that the cross-linking of OX40L *in vitro* results in a dramatic increase in B cell proliferation and secretion of all Ig isotypes (13). This latter effect was independent of a number of investigated cytokines (IL-2, IL-4, IL-5, IL-6, and IL-10) and was due to an increased per cell Ig secretion rate. As explanation for this effect on Ig secretion, we provided evidence that OX40L cross-linking on the B cell surface results in a change in the binding of specific transcription factors to the Ig heavy chain  $\alpha$  enhancer, which results in enhancer activation and increased Ig gene transcription (13, 18).

In the present study, we examined the importance of the OX40-OX40L interaction to a T cell-dependent B cell response *in vivo*. First, we showed that OX40 is maximally expressed on T cells within the PALS 3 d after immunization with TNP-KLH. This peak in OX40 expression falls within the time frame and localization in which the T cell-B cell interaction is believed to take place in the spleen (19). Previous studies (20) have established that the expression of CD40L and the production of several cytokines *in vivo* reach their maximum in the same time period. It should be noted that, whereas the expression of CD40L by activated T cells *in vitro* is only detectable for a very short period of time (21, 22), the expression of OX40 by activated T cells *in vitro* peaks after 24 h and stays upregulated for at least five consecutive days. Although OX40 was not detectable after day 4 *in vivo*, it is possible that it was still expressed but not detectable by our antibody because of an intense interaction with OX40L on activated B cells or the release of soluble OX40L, which either blocks certain OX40 epitopes or leads to an internalization of OX40 molecules on T cells. Similar mechanisms are also believed to render the *in vivo* detection of CD40L by certain antibodies impossible (23, 24).

Second, we demonstrated that OX40-expressing T cells

are in close proximity to specific AFCs in the PALS that have previously been shown to stain positively for OX40L (12). Therefore, an actual interaction through the OX40-OX40L receptor-ligand pair is quite likely.

Third, we showed that this OX40-OX40L interaction is critical for the *in vivo* T cell-dependent humoral immune response. Thus, blocking this interaction with three different systemically administered anti-OX40 antibodies each caused a profound (anti-OX40, anti-P-OX-2) or moderate (anti-P-OX-1) inhibition of the specific IgG response to concomitantly administered antigen (TNP-KLH). This effect was specific for the T cell-dependent B cell response, since the anti-TNP response elicited by immunization with



**Figure 5.** The administration of anti-OX40 inhibits the production of antigen-specific IgG when given during a secondary immunization. 4 mo after the primary immunization with TNP-KLH and the application of rabbit IgG or anti-OX40, BALB/c mice were reimmunized with 100  $\mu$ g i.p. TNP-KLH in IFA. The animals were subsequently not treated at all (*top*) or treated the same way as during the primary immunization (*bottom*). Serum was collected 7 d after the secondary challenge and TNP-specific IgG1 was determined as described in Materials and Methods. Results depict the OD<sub>490</sub> readings for anti-TNP-IgG1. Control animals, which received TNP-KLH in IFA for the first time, produced about three times lower anti-TNP-IgG1 serum levels at day 7 compared to animals immunized for the second time with the same antigen, with the primary immunization consisting of TNP-KLH in CFA (data not shown).

T cell-independent antigens (TNP-Ficoll [T1-2] or TNP-LPS [T1-1]) was not affected by the administration of anti-OX40. Furthermore, the effect of the anti-OX40 antibodies was not due to complement-mediated lysis of T cells, since we did not observe a decrease in cytokine-secreting T cells as assessed by immunohistochemical staining for IL-2, IFN- $\gamma$ , IL-4, and IL-5. This and the fact that germinal center development and IgM response was not impaired indicate that the activated T cells targeted by anti-OX40 were still intact. It is of special interest that the production of specific IgM was not altered by anti-OX40 antibody administration. This could be due to an inhibitory effect of anti-OX40 on Ig isotype switching, but this seems unlikely for several reasons. First, in our prior *in vitro* studies we could not detect any influence of OX40 on isotype switching. In addition, the effect of OX40L cross-linking was observed only after 5 d of culture, considerably after switching had occurred. Finally, if anti-OX40 antibodies inhibit switching, one would expect an increased rather than unchanged IgM antibody response, as seen in patients with a specific genetic defect resulting in a dysfunctional CD40L molecule (hyper-IgM syndrome; 25–28). In view of this, the lack of effect of anti-OX40 antibodies on the production of specific IgM is more likely due to the fact that B cell stimulation through CD40L plus cytokines is sufficient for the production of IgM in the first 6 d after immunization. After that, most of the cells have switched and apparently do need another stimulus for high level Ig secretion, presumably because cytokine expression after day 4 is very low, as shown in previous studies (20, and our unpublished observations).

Finally, we showed that a secondary response to TNP-KLH was not altered by administration of anti-OX40 antibodies during the primary immunization. Although adoptive transfer experiments to formally prove this point have not yet been done, these data suggest that anti-OX40 treatment does not prevent the development of B cell memory. This is in contrast to the results obtained in the studies using an anti-gp39 (anti-CD40L) antibody during primary immunization, since in this case anti-gp39 inhibited both the primary and secondary immune responses (8). However, when we administered anti-OX40 during a secondary immunization, we found that the secondary immune response is also dependent on the OX40–OX40L interaction.

The results obtained by the administration of anti-OX40 antibodies have implications for our current understanding of B cell differentiation *in vivo*. Primary immunization with a T cell-dependent antigen results in two pathways of B cell differentiation: the extrafollicular pathway, which leads to an early antibody response, and the germinal center pathway, in which somatic mutation, memory development, but also plasma cell differentiation, occur (1, 4, 29). A number of authors have suggested that these two pathways are interconnected (3, 19), whereas others have suggested that they are dependent on the presence of distinct B cell precursors, which can be distinguished by their expression of HSA: HSA<sub>low</sub> B cells develop into memory cells, whereas HSA<sub>high</sub> B cells become antibody-producing cells

(10). This theory, however, is opposed by studies by Allman et al. (30), who showed that HSA<sub>high</sub> cells are immature B cells that do not serve as precursors for any particular B cell differentiation pathway. In addition, this theory is at odds with the finding that the same rare CDR3 mutation is shared by cells in the PALS and in the germinal centers (3). Regardless of which of the above mentioned hypotheses is true, the presented data on the effect of anti-OX40 administration suggest that OX40–OX40L interaction is crucial for the development of antibody-secreting cells, independent of their presumed origin.

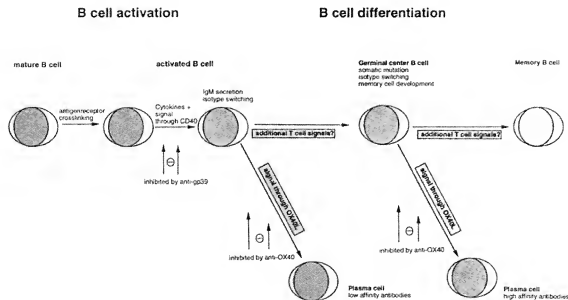
Previous studies that investigated the role of T cell–B cell interaction *in vivo* using antibodies against specific stimulatory surface molecules on the T cell (CD40L) (8) or on the B cell (B7-1/B7-2) (5–7) demonstrated the importance of these receptor–ligand pairs in the early phases of B cell activation *in vivo*. Both early antibody response and germinal center formation—and thereby B cell memory development—were impaired. One previous study reported a selective block of the occurrence of one of the two different B cell differentiation pathways: Pulendran et al. (31) were able to demonstrate that the application of soluble antigen before injection of aggregated antigen inhibits preferentially the appearance of specific germinal centers and B cell memory development. The authors did not offer an explanation for this effect. However, it could be that the application of soluble antigen interferes with the interaction of activated B cells with the antigen–antibody complexes on follicular dendritic cells, which are known to be important for the formation of germinal center (29).

The results of the present study, however, indicate that the OX40–OX40L interaction might be of special importance for the development of antibody-secreting cells but not for the formation of germinal centers. We underlined this theory by demonstrating a reduced development of PALS-associated B cell foci specific for TNP in addition to the almost total abolishment of the specific anti-TNP IgG response. On the other hand, the development of peanut agglutinin-positive, IgD<sup>+</sup> germinal centers remained intact, as did B cell memory differentiation, as suggested in secondary immunization studies.

The present data and data from previous studies allow us to postulate a modified view of the events occurring during the course of a T cell-dependent B cell response, which is summarized in Fig. 6. 2–4 d after primary immunization with a T cell-dependent antigen, B cells interact with CD40L on activated T cells and are also stimulated by T helper 2-type cytokines to secrete IgM and to undergo the initial steps of Ig isotype switching (29, 32). Whereas the CD40L signal is the crucial activation step for Ig isotype switching and subsequent B cell differentiation events (8), the early IgM response does not seem to be totally dependent upon this stimulus, since patients with hyper-IgM syndrome secrete large amounts of IgM after Ig receptor cross-linking and help from cytokines in the absence of a functional CD40L molecule expressed on activated T cells (33). This is also in accordance with *in vitro* studies showing that Ig receptor cross-linking together with, for exam-



# Primary Immunization



**Figure 6.** Model for the role of the OX40–OX40L interaction during the course of T-dependent B cell differentiation in vivo. See Discussion for details.

ple, IL-5 in the absence of CD40L, is sufficient to cause Ig secretion (34).

The next step in B cell differentiation involves the formation of PALS-associated B cell foci and germinal centers. In either case, B cells interact with OX40 on activated T cells, which is critical for high level Ig production, presumably because this interaction leads to an activation of the Ig heavy chain 3' enhancer (13). We do not know at this point if it is (a) sufficient for the cells that form the germinal centers to receive the OX40 signal before they migrate to the follicles, which only becomes apparent after some of the differentiated cells leave the germinal centers and emerge as plasma cells, or if (b) cells leaving the germinal centers to form plasma cells have to receive an additional stimulus through OX40L, as suggested in Fig. 6. This latter possibility cannot be ruled out, because of the long-lasting expression of OX40 on activated T cells in vitro. In any event, the OX40–OX40L interaction cannot be circumvented by cytokines as shown by our in vitro studies and by the fact that the IgG responses are completely abrogated by anti-OX40 administration, which does not result in a decrease of cytokine-producing T cells. It should be noted that this view does not deny the important role of certain cytokines,

especially IL-6, in the terminal differentiation of B cells as demonstrated in IL-6 knockout mice (35). We suggest that these cytokines, which in the case of IL-6 are also produced by activated macrophages and B cells (36), probably act after the necessary OX40 stimulus and drive the B cells into fully differentiated plasma cells, which cannot be accomplished by the cross-linking of OX40L alone (13).

Finally, it is important to add that so far only the OX40–OX40L interaction, which occurs between activated T cells and activated B cells, ensures that activated T cells expressing CD40L do not stimulate B cells through interaction with CD40, which is constitutively expressed on B cells. Thus activated T cells do not have the potential to cause uncontrolled B cell via bystander activation.

In conclusion, we suggest that at least two T cell–B cell surface interactions must occur before the B cell can produce large amounts of IgG antibodies in response to a T cell-dependent antigen. One involving CD40L–CD40 leads to Ig isotype switching and is crucial for all subsequent B cell differentiation events; another involving OX40–OX40L allows B cells to produce large amounts of Ig. Further work defining the role of this second interaction in immunodeficiency and autoimmunity is currently in progress.

The authors thank Dr. David Calderhead (Bristol-Myers Squibb, Seattle, WA) for providing the OX40-Ig fusion protein; Dr. John Inman for his help in the preparation of the TNP conjugates; Dr. John Yewdell for

his support regarding the fluorescence microscopy, and also Dr. David Allman for helpful discussions and critically reading the manuscript.

Dr. Stüber was supported by a grant from the Deutsche Forschungsgemeinschaft (Stu 157/2-1).

Address correspondence to Dr. Warren Strober, Mucosal Immunity Section, Laboratory of Clinical Investigation, National Institutes of Health, Building 10, Room 11-N-238, Bethesda, MD 20892-1890.

Received for publication 6 June 1995 and in revised form 3 November 1995.

## References

- Liu, Y.-L., J. Zhang, P.J.L. Lane, E.Y.-T. Chan, and I.C.M. MacLennan. 1991. Sites of specific B cell activation in primary and secondary responses to T cell-dependent and T cell-independent antigens. *Eur. J. Immunol.* 21:2951-2962.
- Jacob, J., R. Kassir, and G. Kelsoe. 1991. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. I. The architecture and dynamics of responding cell populations. *J. Exp. Med.* 173:1165-1175.
- Jacob, J., and G. Kelsoe. 1991. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. II. A common clonal origin for periaortic lymphoid sheath-associated foci and germinal centers. *J. Exp. Med.* 176:679-687.
- Kelsoe, G., and B. Zeng. 1993. Sites of B-cell activation in vivo. *Curr. Opin. Immunol.* 5:418-422.
- Lane, P., C. Burdet, S. Hubele, D. Scheidegger, U. Müller, F. McConnell, and M. Kosco-Vilbois. 1994. B cell function in mice transgenic for mCTLA-Hg1: lack of germinal centers correlated with poor affinity maturation and class switching despite normal priming of CD4<sup>+</sup> T cells. *J. Exp. Med.* 179:819-830.
- Lintsey, P., P.M. Wallace, J. Johnson, M.G. Gibson, J.L. Green, J.A. Ledbetter, C. Singh, and M.A. Tepper. 1992. Immunosuppression in vivo by a soluble form of the CTLA-4 T cell activation molecule. *Science (Wash. DC)* 257:792-795.
- Ronchese, F., B. Hausmann, S. Hubele, and P. Lane. 1994. Mice transgenic for a soluble form of murine CTLA-4 show enhanced expansion of antigen-specific CD4<sup>+</sup> T cells and defective antibody production in vivo. *J. Exp. Med.* 179:809-817.
- Foy, T.M., J.D. Laman, J.A. Ledbetter, A. Aruffo, E. Claassen, and R.J. Noelle. 1994. gp39-CD40 interactions are essential for germinal center formation and the development of B cell memory. *J. Exp. Med.* 180:157-163.
- Fuleihan, R., N. Ramesh, and R.S. Geha. 1993. Role of CD40-CD40-ligand in Ig-isotype switching. *Curr. Opin. Immunol.* 5:963-967.
- Linton, P.-J., D.J. Decker, and N.R. Klinman. 1989. Primary antibody forming cells and secondary B cells are generated from separate precursor cell populations. *Cell* 59:1049-1059.
- Paterson, D.J., W.A. Jefferys, J.R. Green, M.R. Brandon, P. Corthesy, M. Puklavec, and A.F. Williams. 1987. Antigens of activated rat T lymphocytes including a molecule of 50,000 Mr detected only on CD4 positive T blasts. *Mol. Immunol.* 24:1281-1290.
- Calderhead, D.M., J.E. Buhlmann, A.J.M. van der Eertwegh, E. Claassen, R.J. Noelle, and H.P. Fell. 1993. Cloning of mouse OX40: a T cell activation marker that may mediate T-B interactions. *J. Immunol.* 151:5261-5271.
- Stüber, E.R., M.F. Neurath, D. Calderhead, H.P. Fell, and W. Strober. 1995. Cross-linking of OX40 ligand, a member of the TNF/NGF cytokine family, induces proliferation and differentiation in murine splenic B cells. *Immunity* 2:507-521.
- Bradford, M.M. 1976. A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
- Claassen, E., N. Kors, and N. van Rooijen. 1986. Influence of carriers on the development and localization of anti-trinitrophenyl antibody-forming cells in the murine spleen. *Eur. J. Immunol.* 16:492-497.
- Baum, P.R., R.B. Gayle, F. Ramsdell, S. Srinivasan, R.A. Sorensen, M.L. Watson, M.F. Seldin, E. Baker, G.R. Sutherland, K.N. Clifford, et al. 1994. Molecular characterization of murine and human OX40/OX40 ligand systems: identification of a human OX40 ligand as the HTLV-1 regulated protein gp34. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:3992-4001.
- Godfrey, W.R., F.F. Fagnoni, M.A. Harara, D. Buck, and E.G. Engleman. 1994. Identification of a human OX-40 ligand, a costimulator of CD4<sup>+</sup> T cells with homology to tumor necrosis factor. *J. Exp. Med.* 180:757-762.
- Neurath, M.F., E.E. Max, and W. Strober. 1995. Pax5 regulates the murine immunoglobulin 3' enhancer by affecting binding of NFAP, a protein that controls heavy chain transcription. *Proc. Natl. Acad. Sci. USA* 92:5336-5340.
- van Rooijen, N., W.E. Claassen, and P. Eikelenboom. 1986. Is there a single differentiation pathway for all antibody-forming cells in the spleen? *Immunol. Today* 7:193-196.
- Van den Eertwegh, A.J.M., R.J. Noelle, M. Roy, D.M. Shepherd, A. Aruffo, J.A. Ledbetter, W.J.A. Boersma, and E. Claassen. 1993. In vivo CD40-gp39 interactions are essential for thymus-dependent humoral immunity. I. In vivo expression of CD40 ligand, cytokines, and antibody production delineates sites of cognate T-B cell interactions. *J. Exp. Med.* 178:1555-1565.
- Roy, M., T. Waldschmidt, A. Aruffo, J.A. Ledbetter, and R.J. Noelle. 1993. The regulation of the expression of gp39, the CD40 ligand, on normal and cloned CD4<sup>+</sup> T cells. *J. Immunol.* 151:2497-2510.
- Castle, B.E., K. Kishimoto, C. Stearns, M.L. Brown, and M.R. Kehry. 1993. Regulation of expression of the ligand for ligand on T helper lymphocytes. *J. Immunol.* 151:1777-1788.
- Ling, N.R., I.C.M. MacLennan, and D.Y. Mason. 1987. B cell and plasma cell antigens: new and previously defined clusters. In *Leukocyte Typing III*. A.J. McMichael, editor. Oxford University Press, Oxford. 302-335.
- van Kooten, C., C. Gaillard, J.P. Gallizi, P. Hermann, F. Fossiez, J. Banchereau, and D. Blanchard. 1994. B cells regulate expression of CD40 ligand on activated T cells by lowering the mRNA level and through the release of soluble CD40. *Eur. J. Immunol.* 24:787-792.
- Aruffo, A., M. Farrington, D. Hollenbaugh, X. Li, A. Milatovich, S. Nonoyama, J. Bajorath, L.S. Grossmeier, R. Sten-

- kamp, and R. Neubauer. 1993. The CD40 ligand, gp39, is defective in activated T cells from patients with hyper-IgM syndrome. *Cell*. 72:291-300.
26. Allen, R.C., R.J. Armitage, M.E. Conley, H. Rosenblatt, N.A. Jenkins, N.G. Copeland, M.A. Bedell, S. Edelhoff, C.M. Distche, and A. Simoneau. 1993. CD40 ligand gene defects responsible in X-linked hyper-IgM syndrome. *Science (Wash. DC)*. 259:990-993.
27. DiSanto, J.P., J.Y. Bonnefoy, J.F. Gauchat, A. Fischer, and G. de Saint Basile. 1993. CD40-ligand mutations in x-linked immunodeficiency with hyper-IgM. *Nature (Lond.)*. 361:541-543.
28. Korthauer, U., D. Graf, H.W. Mages, F. Briere, M. Padayachee, S. Malcom, A.G. Ugazio, L.D. Notarangelo, R.J. Levinsky, and R.A. Kroccek. 1993. Defective expression of T-cell CD40 ligand causes x-linked immunodeficiency with hyper-IgM. *Nature (Lond.)*. 361:539-541.
29. MacLennan, I.C.M. 1994. Germinal centers. *Annu. Rev. Immunol.* 12:117-139.
30. Allman, D.M., S.E. Ferguson, V.M. Lentz, and M.P. Cancro. 1993. Peripheral B cell maturation. II. Heat-stable antigen(hs) splenic B cells are an immune developmental intermediate in the production of long lived marrow-derived B cells. *J. Immunol.* 151:4431-4444.
31. Pulendran, B., M. Karvelas, and G.J.V. Nossal. 1994. A form of immunologic tolerance through impairment of germinal center development. *Proc. Natl. Acad. Sci. USA*. 91:2639-2643.
32. Weinstein, P.D., and J.J. Cebra. 1991. The preference for switching to IgA by Peyer's patch germinal center B cells is likely due to the intrinsic influence of their microenvironment. *J. Immunol.* 147:4126-4135.
33. DiSanto, J.P., G. de Saint Basile, A. Durandy, and A. Fischer. 1994. Hyper-IgM syndrome. *Rev. Immunol.* 145:205-209.
34. Pecanha, L.M.T., H. Yamaguchi, A. Lees, R.J. Noelle, J. Mond, and C.M. Snapper. 1993. Dextran-conjugated anti-IgD antibodies inhibit T cell-mediated IgE production but augment the synthesis of IgM and IgG. *J. Immunol.* 150:2160-2168.
35. Ramsay, A.J., A.J. Husband, I.A. Ramshaw, S. Bao, K.I. Matthei, G. Koehler, and M. Kopf. 1994. The role of interleukin-6 in mucosal IgA antibody responses in vivo. *Science (Wash. DC)*. 264:561-563.
36. Chen-Kian, S. 1995. Regulation of terminal differentiation of human B cells by IL-6. *Curr. Top. Microbiol. Immunol.* 194: 189-198.